The Journal of Microbiology, June 2004, p.69-73 Vol. 42, No. 2

Changes in Membrane Fatty Acid Composition during Entry of *Vibrio vulnificus* into the Viable But Nonculturable State

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(Received March 9, 2004 / Accepted May 20, 2004)

***Vibrio vulnificus*, a Gram-negative bacterium found in estuarine waters, is responsible for over 95% of all seafood-related deaths in the United States. As a result of a temperature downshift to 5°C, this organism enters the viable but nonculturable (VBNC) state. Changes in the membrane fatty acid (FA) composition of *V. vulnificus* may be a contributing factor to the ability of this organism to enter into and survive in the VBNC state. This hypothesis was tested by incubating the organism at 5°C in artificial sea water and analyzing the cells' FAs during the initial hours of temperature and nutrient downshift. Prior to downshift, the predominant FAs were 16:0, 16:1 and 18:0. During the first four hours of downshift, statistically significant changes occurred in 15:0, 16:1, 16:0, 17:0, and 18:0. These results indicate that changes in FA composition occur prior to entry of *V. vulnificus* into the VBNC state, suggesting that the ability to maintain membrane fluidity may be a factor in this physiological response. Cells in which fatty acid synthesis was inhibited did not survive, indicating that active fatty acid metabolism is essential for entry of cells into the VBNC state.**

***Key words*:** *Vibrio vulnificus, fatty acids, VBNC, culturability*

Vibrio vulnificus is an estuarine bacterium which accumulates to high levels in filter-feeding shellfish. This bacterium is a serious human pathogen, responsible for greater than 95% of all seafood-related deaths in the United States (Oliver and Kaper, 2001). Infection by *V. vulnificus* can manifest in three different types of disease in humans: serious wound infections, a potentially fatal septicemia, and gastroenteritis (Oliver, 1989; Oliver and Kaper, 2001). Wound infections due to *V. vulnificus* occur when a pre-existing or newly inflicted wound is exposed to estuarine water containing the bacterium. Severe necrosis of the tissue surrounding the wound occurs, often requiring debridement or amputation of the affected areas. Primary septicemia results from ingestion of the bacterium in raw or undercooked seafood, typically oysters and clams (Oliver, 1989; Oliver and Kaper, 2001). However, unlike wound infections, the individual generally must have some pre-existing health condition which predisposes to the bacterium. Risk factors associated with primary septicemia include being male over the age of forty, having liver disorders and/or being immunocompromised. Cases of *V. vulnificus* gastroenteritis following ingestion of shellfish are relatively uncommon, and typically result in diarrhea and abdominal cramping (Oliver and Kaper, 2001).

V. vulnificus is frequently cultured from the environment during warm months, however it is extremely difficult to isolate during winter months (Oliver, 1993). This seasonality is due to entry of *V. vulnificus* into the viable but nonculturable (VBNC) state when water temperatures are below ca. 10°C. In this physiological state, the bacterial cells are unable to grow on routine bacteriological media, although they retain viability (Oliver, 1993). During entry into the VBNC state, cells characteristically become smaller in size and drastically decrease production of macromolecules (Oliver *et al.*, 1991; Oliver, 2000). Over 40 other bacterial species, including *Escherichia coli* and several *Salmonella* and *Pseudomonas* spp. have also been shown to enter the VBNC state as a response to factors such as temperature fluctuations, light, elevated salinity, and nutrient starvation (Oliver, 2000).

The bacterial cell membrane is essential in responding to changes in the environment such as temperature downshifts and modifications in osmotic concentration (Denich *et al.*, 2003). Membrane fluidity is regulated through fluctuations of saturation and chain lengths of phospholipid fatty acids, permitting the membrane to function under different environmental conditions (Magnuson *et al.*, 1993). As temperature decreases, the percentage of unsaturated fatty acids in the bacterial membrane increases, thus lowering the melting point of the lipids (Fujii and Fulco, 1977; Silvius *et al.*, 1980). A shift in fatty acids to shorter chain lengths provides similar compensation.

While the VBNC state of *V. vulnificus* and other bac-

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teria is now extensively documented, the biochemical events responsible remain unclear. Because specific changes occur in the fatty acid composition of bacterial membranes during a temperature downshift, it is likely that such changes contribute to the viability of *V. vulnificus* at 5°C, thus allowing the bacterial cells to enter the VBNC state. A study of the membrane fatty acid composition of *V. vulnificus* cells during the first hours of temperature downshift could reveal changes that allow a bacterial cell to remain viable at decreased temperatures.

Materials and Methods

Bacterial Strains and Microcosm Preparation

V. vulnificus strain C7184 (opaque) was employed in all studies and was routinely grown overnight at ca. 22°C in HIA (Heart infusion broth with artificial sea water [ASW] as the diluent). A 1% inoculation of the overnight culture was then added to HIA broth and grown to log phase (OD_{610}=0.15-0.20). The cells were washed with ASW, resuspended in 1 ml of ASW, and added to 99 ml of ASW. These microcosms were then placed at 5°C to induce the VBNC state.

Quantification of Cells

Culturable cell counts following serial dilutions in ASW were made on HI agar at each sampling time. Once plate counts indicated a culturable cell density below 10 CFU/mL, the cells were considered to be nonculturable. At that point, viable and total cell numbers were determined by the method of Kogure *et al.* (1979). Briefly, a sample of the microcosm (0.9 ml) was removed and 0.1 ml of a 0.25% yeast extract (Difco, USA) and 0.02% nalidixic acid (Sigma, USA) solution was added to the sample. The cells were incubated overnight at room temperature, fixed with filter-sterilized 37% formalin and stained with 0.01% acridine orange. Samples were filtered onto a 0.2 μm black polycarbonate filter (Poretics, USA) and viewed using epifluorescence microscopy (Olympus BH2-RFCA). If a cell was viable, it would elongate in response to the yeast extract, but be unable to divide due to the inhibition of cell wall septation by nalidixic acid. Therefore, elongated cells (at least 2× the length of control cells) were considered to be viable. A control sample lacking Kogure reagents was employed to determine spontaneous elongation, and these values were subtracted from experimental data points.

Inhibition of Fatty Acid Synthesis

Cerulenin (Sigma, USA) was dissolved in 95% ethanol and 100 μg of the antibiotic was added per mL of early-exponentially growing cells (OD_{610}= 0.09-0.10). Cells were allowed to incubate for a further 30 min. Cells were then washed and resuspended in ASW as described above.

Lipid Analysis

Membrane phospholipids were extracted by the Bligh-Dyer method (1959). A standard method of transesterification using BF_3-methanol (Supelco, USA) was employed to hydrolyze and methylate the phospholipid fatty acids (Metcalfe and Schmitz, 1961). Following removal and drying of the organic layer, carbon disulfide was added and the sample was transferred to Teflon-capped glass tubes for storage.

Bacterial fatty acid methyl esters (FAMEs) were analyzed by gas chromatography employing a flame ionization detector. FAMEs were separated in a DC200 column (6 length, 1/8" diameter, 10% chromWCW DMCS; Supelco, USA) using ultra high-purity nitrogen as the carrier gas. The settings were as follows: injector temperature 200°C, detector temperature 200°C, column temperature 180°C, carrier gas flow rate 34 ml/min. Peak retention times and areas were calculated by the retention time × peak height method, with chain lengths and saturations determined by comparison to authentic standards. The results were expressed as percentages of total fatty acids.

Statistical Analysis of Data

Each experiment was repeated three or four times, except for the study on fatty acid changes occurring in the *V. vulnificus* membrane over an extended time period. That study was performed once. Numerical data for the fatty acid membrane composition studies represent the standard error of the mean. In each experimental set-up (HIA or HIA/cerulenin), statistical differences for percent fatty acids between times zero and four hours of temperature downshift were determined by a one-way analysis of variance (ANOVA) followed by the Student-Newman-Kuels *post hoc* test. Results were considered significant for $p<0.05$.

Results

V. vulnificus grown in HIA and downshifted to 5°C

Under the conditions employed in our studies, microcosms reached a temperature of 5°C by ca. 2.5 h after being placed at this temperature (Fig. 1A). Culturable cell numbers remained constant during this time (Fig. 1A), but cells were no longer culturable by 30-40 days (Fig. 1B). At that time, however, direct viability assays indicated that *ca.* 8.5×10^4 cells/mL retained viability. These results indicate that a large population of cells existed in the VBNC state.

Analysis of the fatty acids of these cells showed that hexadecanoic acid (16:0), hexadecenoic acid (16:1) and octadecanoic acid (18:0) were the predominant fatty acids (ca. 80%) prior to temperature downshift (Fig. 2). Statistically significant changes occurred during the first four hours of temperature downshift in pentadecanoic acid (15:0), hexadecenoic acid (16:1), heptadecanoic acid

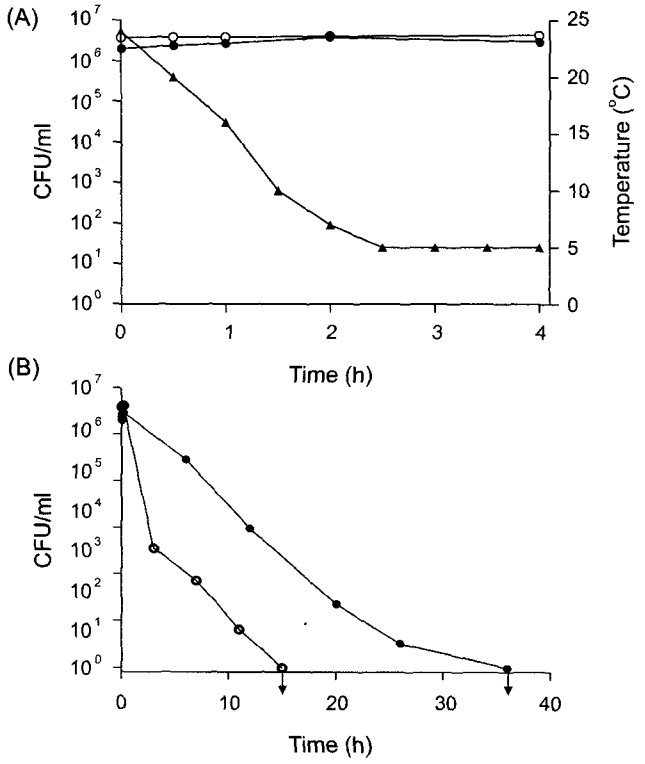

Fig. 1. Microcosm temperature and *V. vulnificus* culturability. (A) Temperature of the ASW microcosm during the first four hours of temperature downshift to 5°C (▲) and culturability of cells incubated in ASW at 5°C with (○) and without (●) the fatty acid synthesis inhibitor, cerulenin. (B) Long-term culturability of *V. vulnificus* at 5°C. Cells in ASW (●) or ASW+cerulenin (○). Arrows indicate where culturability was below the limit of detection.

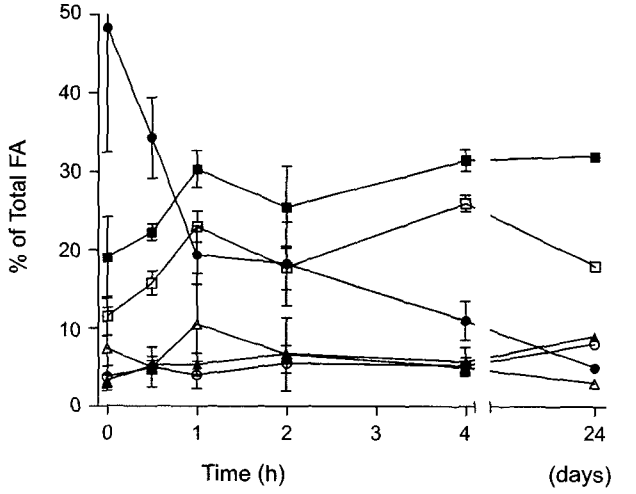

Fig. 2. Change in membrane fatty acid composition of *V. vulnificus* in ASW during temperature downshift. The fatty acids seen are hexadecanoic acid (16:0, ●), hexadecenoic acid (16:1, ■), octadecanoic acid (18:0, □), heptadecanoic acid (17:0, ▲), tetradecanoic acid (14:0, ○), and octadecenoic acid (18:1, △). For clarity, 15:0 and 15:1, both of which were ca. 5% of the total fatty acids, are not illustrated. Error bars indicate standard error of means of four replicate studies except for the single point at 24d. Significant ($p<0.05$) changes over the initial 4 h study period were found for 15:0, 16:0, 16:1, 17:0, and 18:0.

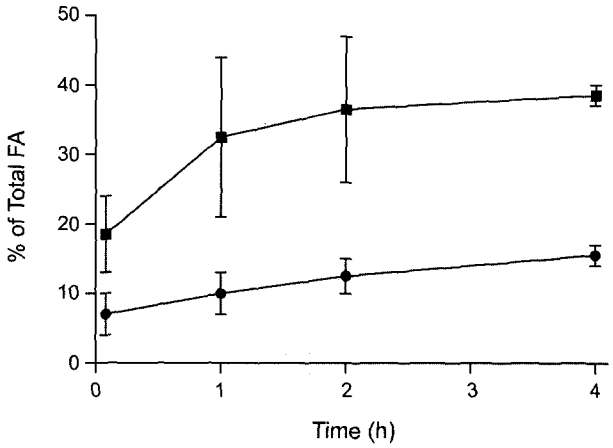

Fig. 3. Percentage of unsaturated membrane fatty acids (■) and of fatty acids with chain lengths less than 16 carbons (●) of *V. vulnificus* during the first four hours of temperature downshift. Error bars indicate standard error of means of three replicate studies. Significant ($p<0.05$) changes over the initial 4 h study period existed in both measurements.

(17:0) and octadecanoic acid (18:0), with the greatest change seen in hexadecanoic acid (16:0) (Fig. 2). Changes in pentadecenoic acid (15:1) approached statistical significance. A significant increase in the percentage of unsaturated fatty acids and in fatty acids with chain lengths less than 16 carbons occurred during this time (Fig. 3).

Long-term incubation of HIA-grown cells at 5°C

After cells had remained in ASW at 5°C for 24 days, the major fatty acids observed were hexadecenoic acid (16:1) and octadecanoic acid (18:0) (Fig. 2). Hexadecanoic acid (16:0) dropped drastically during this time, from nearly 50% of the total fatty acid composition prior to temperature downshift to 5% at day 24.

Effect of cerulenin on fatty acid synthesis during temperature downshift

Cerulenin-treated cells became nonculturable at ca. 15 days of temperature downshift to 5°C (Fig. 1B). Direct viability assays at this point indicated that the cells were no longer metabolically active and had presumably died. Analysis of the membrane fatty acid composition during the first four hours of temperature downshift of these cells (Fig. 4) revealed that no significant changes occurred in any of the fatty acids, nor in degree of saturation or chain length (data not shown).

Discussion

Fatty acid composition of V. vulnificus *during initial temperature downshift*

In this study, the predominant fatty acids of *V. vulnificus* grown in HIA were found to be 16:0, 16:1, and 18:0. These results are similar to previous studies on the fatty acid composition of *V. vulnificus* (Linder and Oliver,

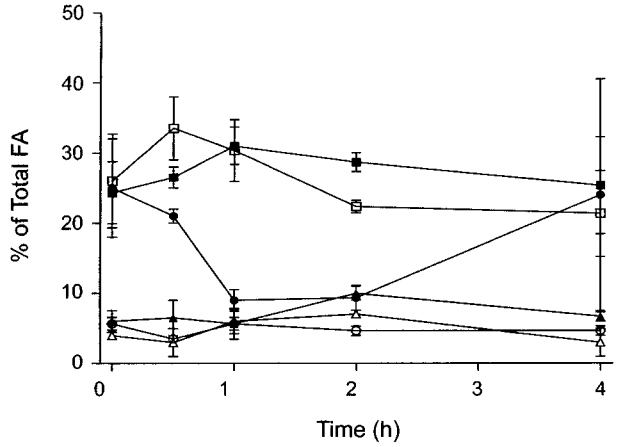

Fig. 4. Change in membrane fatty acid composition of cerulenin-treated *V. vulnificus* in ASW during temperature downshift. The fatty acids seen are as indicated in Fig. 2. Error bars indicate standard error of means of three replicate studies.

1989; Bertone *et al.*, 1996; Shin *et al.*, 1997). Other studies (e.g. Oliver and Colwell, 1973; Lambert *et al.*, 1983) also reported 16:0 and 16:1 to be the predominant fatty acids in this genus, although 18:1 instead of 18:0 is usually found to be a major fatty acid, and psychrophilic vibrios from deep-sea environments frequently contain polyunsaturated fatty acids (e.g. Hamamoto *et al.*, 1994; Jøstensen and Landfald, 1996). The difference in the 18/18:1 levels may be due to the high osmotic concentration of the growth medium we employed (HI with ASW as diluent).

Since the cells in our study were experiencing starvation stress as well as a temperature downshift, it is possible that the changes seen in fatty acid composition could have been due to the effects of starvation as well as low temperature incubation. Our results, however, especially the increase in 16:1, are in contrast to those of a starvation study reported by Oliver and Stringer (1984). That study, on a psychrophilic *Vibrio* species, reported a decrease in 16:1 during starvation, while other fatty acids showed little change. This conclusion is also supported by a fatty acid study reported by Rice and Oliver (1992), who found no change in 16:1 during starvation of marine barophile, CNPT-3. Therefore, while the fatty acid changes we observed as the cells entered the VBNC state are primarily a result of the temperature downshift the cells experienced, it is also possible that our results reflect the combined effects of temperature and nutrient downshift.

Our study found that the temperature in ASW microcosms reached 5°C by ca. two hours of temperature downshift (Fig. 1A). Therefore, changes in membrane composition would be expected to occur prior to four hours of temperature downshift, and indeed we observed large changes, especially in 16:0, during the first hour of low temperature incubation. An examination of the fatty acid content after an extended period (24 days) at 5°C suggested that the bacterium did not greatly alter its fatty acids after the initial few hours (Fig. 2).

At the initiation of our studies, the predominant fatty acids observed were 16:0, 16:1 and 18:0, with the amount of 16:0 dramatically decreasing during incubation at 5°C. These results are similar to those reported by Linder and Oliver (1989) on *V. vulnificus* during entry into the VBNC state, except that those investigators also observed a drastic decrease in 16:1. The most likely explanation for this difference is the fact that the study used HI broth, rather than HIA broth, as the growth medium prior to introduction of the cells into ASW microcosms. Their study, therefore, involved not only a temperature and nutrient downshift, but a significant osmotic upshift as the cells were moved from HI (300 mOsm) to ASW (920 mOsm). Further, their first sample point was not taken until 14 days into the temperature downshift, at which time most fatty acid changes would likely have already taken place.

Effect of inhibition of fatty acid synthesis on viability and fatty acid composition

Cerulenin is an antibiotic that inhibits *de novo* fatty acid synthesis through inhibition of the enzyme, fatty acid synthase (Dees and Oliver, 1977; Ehlert and Höltje, 1996). By adding this antibiotic to the bacterial cells prior to temperature downshift, the cells were prevented from synthesizing new fatty acids in response to the downshift. The overall fatty acid pattern of cerulenin-treated cells showed no significant changes over the 4 h time period (Fig. 4), indicating that cerulenin did indeed inhibit *de novo* fatty acid synthesis. Further, the fact that the entire population was dead *ca.* 15 days after temperature downshift suggests that changes in membrane fatty acids and their effect on maintenance of membrane fluidity in response to temperature downshift are essential for survival of this bacterium at low temperature. As this study showed that cells would otherwise enter the VBNC state when placed at 5°C, death of cerulenin-treated cells is evidently related to their inability to synthesize fatty acids and alter membrane fluidity.

In conclusion, this study reports changes that occur in the membrane fatty acids of *V. vulnificus* during a temperature downshift. We believe these changes permit the cells to maintain membrane fluidity, thus allowing maintenance of viability at low temperatures. In contrast, if the cells are prevented from altering their membrane fatty acid composition (*e.g.* by cerulenin treatment), viability and the ability to enter the VBNC state are lost.

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